

Estrogen and Androgen Receptor Activities of Hydraulic Fracturing Chemicals and Surface and Ground Water in a Drilling-Dense Region

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The rapid rise in natural gas extraction using hydraulic fracturing increases the potential for contamination of surface and ground water from chemicals used throughout the process. Hundreds of products containing more than 750 chemicals and components are potentially used throughout the extraction process, including more than 100 known or suspected endocrine-disrupting chemicals. We hypothesized that a selected subset of chemicals used in natural gas drilling operations and also surface and ground water samples collected in a drilling-dense region of Garfield County, Colorado, would exhibit estrogen and androgen receptor activities. Water samples were collected, solid-phase extracted, and measured for estrogen and androgen receptor activities using reporter gene assays in human cell lines. Of the 39 unique water samples, 89%, 41%, 12%, and 46% exhibited estrogenic, antiestrogenic, androgenic, and antiandrogenic activities, respectively. Testing of a subset of natural gas drilling chemicals revealed novel antiestrogenic, novel antiandrogenic, and limited estrogenic activities. The Colorado River, the drainage basin for this region, exhibited moderate levels of estrogenic, antiestrogenic, and antiandrogenic activities, suggesting that higher localized activity at sites with known natural gas-related spills surrounding the river might be contributing to the multiple receptor activities observed in this water source. The majority of water samples collected from sites in a drilling-dense region of Colorado exhibited more estrogenic, antiestrogenic, or antiandrogenic activities than reference sites with limited nearby drilling operations. Our data suggest that natural gas drilling operations may result in elevated endocrine-disrupting chemical activity in surface and ground water. (*Endocrinology* 155: 897–907, 2014)

Hundreds of synthetic and naturally occurring chemicals have the ability to disrupt normal hormone action and have been termed endocrine-disrupting chemicals (EDCs). EDCs can act through multiple mechanisms: direct interaction with hormone receptors (1, 2), indirect enhancement or suppression of a receptor's ability to respond to endogenous hormones (3, 4), or modulation of endogenous hormone levels (4, 5). EDCs are different from toxicants in that they have been shown to exhibit nonmonotonic dose-response curves, resulting in quantitatively and qualitatively different health outcomes at low

vs high doses. Laboratory experiments have shown a wide range of effects at environmentally relevant, low concentrations that were not predicted by traditional risk assessments from high-dose testing (6–9). EDCs may be of particular concern during critical windows of development when exposure can alter normal development and has been linked to adult disease (6, 9).

EDCs have been measured in humans and other animals, and exposure has been linked to a number of negative health effects (9–11). Although EDCs have been described to disrupt many hormone systems, chemicals that

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Abbreviations: CMV, cytomegalovirus; EDC, endocrine-disrupting chemical; Gal, galactosidase; MEM, minimal essential medium.

disrupt estrogen and androgen receptor action have documented health outcomes at environmentally relevant exposure levels. Exposure to estrogenic chemicals has been linked to decreased fertility, increased cancer incidence, impaired gonadal development, and more (2, 12, 13). Exposure to antiandrogenic chemicals has been linked to decreased sperm quality and quantity, delayed preputial separation, hypospadias and cryptorchidism, decreased anogenital distance (a biomarker for fetal androgen exposure), reproductive tract deformities, and other adverse health outcomes (14–17). Exposure to antiestrogenic chemicals may be the least understood, although research on ewes in pastures treated with sewage sludge exhibited reduced bone density and mineral content, endpoints that have been reported with exposure to antiestrogens (18).

A potential novel source of EDCs is through their use in hydraulic fracturing operations for natural gas and/or oil extraction processes. Hydraulic fracturing involves the underground injection of several million gallons of water combined with chemicals and suspended solids (proppants) into each well under high pressure. More than 750 chemicals are reportedly used throughout this process. Of these, more than 100 are known or suspected EDCs, and still others are toxicants and/or carcinogens (19, 20). The rapid expansion in drilling operations using hydraulic fracturing increases the potential for environmental contamination with the hundreds of hazardous chemicals used (20, 21). Importantly, hydraulic fracturing was exempted from multiple federal regulatory acts in 2005 including the Safe Drinking Water Act, the Clean Water Act, and the Clean Air Act (21).

Chemicals are added throughout the drilling and fracturing processes for a variety of reasons. For example, during drilling they are used to reduce friction and shorten drilling time (21, 22). In horizontal or directional wells, drilling starts vertically and then turns and proceeds for up to a mile or more. After stabilization, several million gallons of water, chemicals, and proppants are injected into the well under high pressure to form and maintain fractures throughout the shale or coal bed layer to liberate natural gas and/or oil. Chemicals are injected for reasons ranging from increasing the viscosity to serving as antibacterial agents (22, 23). Once the water mixture has been forced into the well under high pressure, up to 40% may be immediately recovered as flow back, containing the chemicals used for fracturing as well as some naturally occurring chemicals from the shale layer (22). The produced water is composed of naturally occurring compounds from the shale formation and the remaining hydraulic fracturing fluids that come to the surface over the life of a producing well. It should be noted that both of these types of wastewater can be heavily laden with nat-

urally occurring radioactive compounds, heavy metals from the shale layer, and chemicals used in fracturing operations (22, 24) and may be injected into disposal wells, reused in drilling operations, or pumped into open evaporation pits (21, 22).

There have been many reports of changes in surface, ground, and drinking water quality near natural gas drilling operations, particularly in drilling-dense regions, with some specifically linked to natural gas extraction (21, 25, 26). For example, in a 2011 draft report, the US Environmental Protection Agency concluded that chemicals used in natural gas operations had contaminated ground water and domestic water supply in Pavillion, Wyoming (25).

There are many pathways for chemicals used in natural gas operations to contaminate surface and ground waters: spills during transport before and after extraction, the drilling and fracturing processes, disposal of wastewater, failure of well casings, and structural issues surrounding abandoned wells (27, 28). Multiple researchers have demonstrated that levels of stray gases and heavy metals in drinking water increased with proximity to natural gas wells, suggesting the possibility of underground migration of fluids associated with hydraulic fracturing (29–31). Vengosh and colleagues (32) further reported natural connectivity between shallow drinking water aquifers and formations deep underground in areas of the Marcellus Shale, suggesting a route for the potential migration of natural gas drilling fluids into ground water. These studies support the hypothesis that fracturing fluids remaining underground have the potential to migrate into shallow ground water sources over time. Taken together, there is the potential for surface and ground water contamination throughout the entire extraction process.

The goals of this study were 2-fold. First, we measured the estrogenic, antiestrogenic, androgenic, and antiandrogenic activities of 12 suspected or known EDCs used in natural gas operations. Second, we measured the same activities in surface and ground water from a natural gas drilling-dense region in Garfield County, Colorado (Figure 1), an area with approximately 10 444 active wells (33). Of particular concern with exposure to EDCs is the potential for additive effects of mixtures of chemicals that act through a common biological pathway, even when each chemical in the mixture is present at levels below an observed effect threshold (17, 34, 35). Thus, several researchers have taken the approach of measuring the total bioactivity of chemicals with a common mechanism of action in water samples (36, 37). This approach leads to a greater sensitivity of detection because multiple chemicals with the same mechanism of action have additive effects, which are very relevant in the detection of potential contamination of water with hundreds of chemicals at low

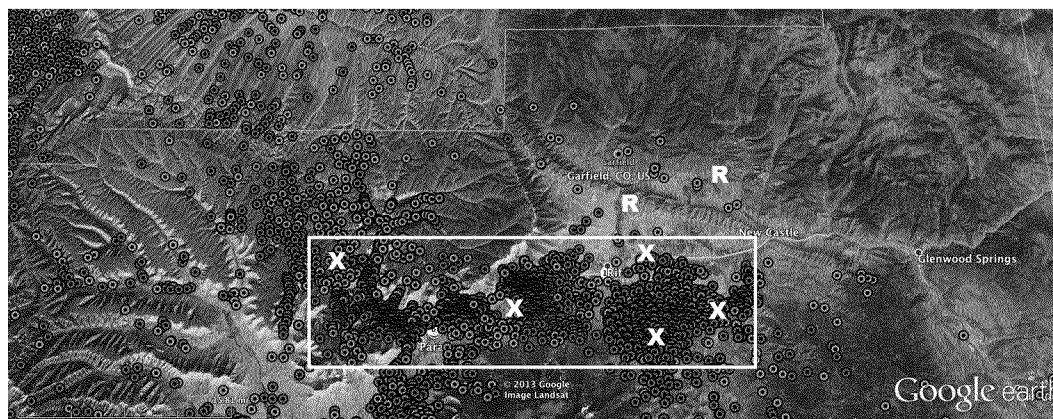


Figure 1. Map of Garfield County sample collection area. Pictorial representation of the sample collection area in Garfield County, Colorado. White rectangle denotes the zone from which all high-density sample collection sites (sites 1–5) were collected. X marks denote high-density drill sites that had also experienced a drilling-related spill, and R marks denote local reference sites outside of the high-density drilling area. Red, orange, and yellow circles denote natural gas drilling wells in various stages of operation as of June 2008. This map represents an underestimation of the wells present when our samples were collected in September 2010. (Map data from Google, Image Landsat; tabulation and mapping of Colorado Oil and Gas Commission data on wells active as of June 2008 from SkyTruth.)

concentrations. We hypothesized that (1) a subset of chemicals used in natural gas operations would exhibit estrogen and/or androgen receptor activity and (2) surface and ground water in this natural gas drilling–dense area, affected by drilling-related spills, would exhibit greater estrogen and androgen receptor activities than reference sites with no or limited drilling activities.

Materials and Methods

Chemicals

17 β -Estradiol (98% pure), ICI 182,780 (Fulvestrant, 98% pure), testosterone (98% pure), flutamide (100% pure), and all other chemicals were purchased from Sigma-Aldrich. Stock solutions were prepared in HPLC-grade methanol (catalog no. A452-1; Thermo Fisher Scientific) at 10 mM and stored at 4°C. The 12 chemicals used in natural gas operations that were selected (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at <http://end.endojournals.org>) were chosen from lists of all known chemicals used in natural gas operations (19, 20), narrowed by selecting only chemicals that were known or suspected EDCs (20), those reportedly used in Colorado, and preference given to chemicals used in multiple chemical products.

Sample collection

All samples were collected in 1-L amber glass bottles (catalog no. 12-100-130; Thermo Fisher Scientific) and certified to meet the US Environmental Protection Agency standards for metals, pesticides, volatiles, and nonvolatiles. Surface water samples were taken from water that had collected on the ground such as rivers, creeks, and ponds and were collected by submerging bottles approximately 10 inches. Ground water samples were taken from water that had collected underground, typically accessed via drinking or monitoring wells. Artesian water samples were defined as ground water sources that had flowed to the surface under pressure and were collected where they met the surface.

Samples were collected by filling bottles 2 times from the source before keeping the third collection. Samples were stored on ice in the field, stored at 4°C in the laboratory, and processed within 2 months of collection. All analyses were performed blinded to sample identification using a unique 6-digit bottle identification.

Reference control sites

Ground water reference samples were collected from one drilling absent location in Boone County, Missouri (Missouri reference) in 2011 and two drilling-sparse (ffl2 wells within 1 mile) locations in Garfield County, Colorado (Colorado reference) in February 2013 within the bounds of the Piceance Shale Basin (Figure 1, Table 1, and Supplemental Table 2). Surface water reference samples were collected from 2 drilling absent locations in Boone County, Missouri (Missouri reference), in 2011. Surface water reference samples from drilling-sparse locations in Garfield County were not obtained because of the scarcity of surface water sources not affected by nearby drilling operations.

Sample sites

Water samples were collected from ground, surface, and artesian (n ffl 9, 19, and 1, respectively) water sources in September 2010 in drilling-dense areas of Garfield County, Colorado, from 5 distinct sites with unique characteristics (Figure 1, Table 1, and Supplemental Table 2). All sites were located within the Colorado River Drainage Basin and the Piceance Shale Basin, had been directionally fractured to extract natural gas, contained from 43 to 136 natural gas wells within 1 mile (Table 1), and a spill or incident related to natural gas drilling processes had occurred within the past 6 years. Five surface water samples were also collected from the Colorado River, the drainage basin for this drilling-dense region.

Process controls

Process controls were prepared using 1 L of HPLC-grade water (catalog no. WFSK-4; Thermo Fisher Scientific) following the same procedure used for all experimental samples. These controls were included in all assays to measure any background

Table 1. Description of Sample Collection Sites

Site No.	No. of Samples Collected	No. of NGD Wells Within 1 Mile ^a	Distance to Colorado River, mi	Approximate Well Depth, ft ^b	Approximate Frack Fluid Volume, gal ^b	Description of Incident	Date of Incident ^c
Missouri reference	3	0	NA				
Colorado reference	2	ffl2	4.75–6.5	Unknown	Unknown		
1	8	43	5.25	5500	4 000 000	Natural gas upwelling	May 2008 ^c
2	8	78	0.75	8000	1 500 000	Fluid spill into creek	December 2009
3	5	69	8.75	9500	1 000 000	Spill at nearby drill pad	May 2008 ^c
4	8	136	6.00	9000	4 000 000	Produced water tank leak	November 2004
5	9	95	0.50	7500	3 000 000	Produced water line leak	July 2010 ^c
Colorado River	5	Varied	NA				

Abbreviations: NA, not applicable; NGD, natural gas drilling.

^a Uses a radius of 1 mile from the sampling location. The number is approximate based on data obtained from the Colorado Oil and Gas Conservation Commission (<http://dnrwebmapgdev.state.co.us/mg2012app/>; accessed April 19, 2012).

^b Information on well depth and typical fracturing fluid volume was obtained from FracFocus based on wells after January 1, 2011, for the same radius as used for well number determination. All samples were collected in September 2010.

^c Documented benzene levels exceeding acceptable limits detected in water tests conducted on or around this date.

hormonal activity contributed by the solid-phase extraction process.

Extraction of water samples

Water samples (1-L) were filtered through a ceramic Buchner funnel using filter paper (90 mm, catalog no. 54; Whatman) to remove suspended solids and were then subjected to solid-phase extraction using Oasis HLB glass cartridges (catalog no. 186000683; Waters) (38). All additions to the cartridges were made using disposable borosilicate glass pipets. Cartridges were attached to a vacuum manifold and conditioned with 100% HPLC-grade methanol and 100% HPLC-grade H₂O. Water samples were loaded onto the cartridge and washed with 5 mL of 5% methanol. They were then removed from the manifold and seated on amber glass vials, where elution was performed with three 1-mL additions of 100% methanol. Eluted samples were then dried under nitrogen and reconstituted in 250 μ L of methanol (100%), creating stock concentrations of 4000ffl the original water concentration. Reconstituted samples were stored at 4°C and protected from light until tested. To be applied to cells, stock samples were diluted 100- and 1000-fold in tissue culture medium, creating final concentrations, in contact with the cells, of 40ffl and 4ffl the original water concentration.

Extraction method recovery efficiencies

Extraction method recovery efficiencies were determined using [³H]17ffiestradiol (100 Ci/mmol; PerkinElmer), [³H]testosterone (70 Ci/mmol; PerkinElmer), and [³H]bisphenol A (7.3 Ci/mmol; Moravak Biochemicals). Tritiated chemicals were spiked at an activity of 1 μ Ci each in 1 L of water and processed in the manner described above. Final concentrations of test chemicals used included 1.4 pM testosterone, 1.4 pM 17ffiestradiol, and 140 pM bisphenol A. Radioactivity was measured for duplicate samples using a scintillation counter before processing, after elution, and after dry-down and reconstitution. Recovery was 71.5% \pm 3.5% for [³H]17ffiestradiol, 79.0% \pm 3.6% for [³H]testosterone, and 71.1% \pm 4.1% for [³H]bisphenol A.

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Cell culture

HepG-2 cells (HB-8065; American Type Culture Collection) were maintained in minimum essential medium (MEM) (Gibco) supplemented with 8% fetal bovine serum (catalog no. SH30396.03; Thermo HyClone), 2 mM GlutaMAX, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. MCF-7 cells (HTB-22; American Type Culture Collection) were maintained in MEM supplemented with 5% newborn calf serum (catalog no. SH30118.03; Thermo HyClone), 2 mM GlutaMAX, 0.1 mM nonessential amino acids, and 6 ng/mL bovine insulin. Water sample and chemical dilutions were performed in the respective media as described above with the following exceptions: the medium used was phenol red-free and sera were charcoal-stripped to remove endogenous steroids. Cell lines were transferred to this modified medium 2 days before the start of assays.

Plasmids

For androgenic activity testing, HepG-2 cells were transfected with androgen receptor, pSG5-AR (39), androgen response element linked to the firefly luciferase gene, 2XC3ARETKLuc (laboratory of Donald P. McDonnell), and cytomegalovirus (CMV)-ffl-galactosidase (Gal) (40). For antiandrogenic activity testing, HepG-2 cells were transfected with androgen receptor, CMV-AR1 (41), androgen response element linked to the firefly luciferase gene, PSA-Enh E4TATA-luc (42), and CMV-ffl-Gal

(40). For estrogenic and antiestrogenic activity testing, MCF-7 cells were transfected with estrogen response element linked to the firefly luciferase gene, 3XERETKLuc (43), and CMV-ffi-Gal (40).

Estrogen and androgen receptor reporter gene assays

Activities were measured using reporter gene assays containing a hormone response element linked to luciferase. Each treatment concentration for each sample was performed in quadruplicate within each assay, and each assay was repeated three times. Cells were cotransfected with the vectors listed above using MEM with reduced serum (catalog no. 31985; Invitrogen). Cells were transfected in T25 or T75 flasks for approximately 5 hours using Lipofectamine LTX and Plus Reagent (catalog no. 15338-100; Invitrogen) and then allowed to recover overnight. Transfected cells were then trypsinized, seeded into 96-well tissue culture plates at approximately 70 000 cells per well, and allowed to settle for 4 hours before induction. Cells were induced with dilution series of the positive/negative controls, the reconstituted water samples at 4ffl and 40ffl concentrations, or a dilution series of the selected subset of chemicals from 10^{-1} M to 10 nM, diluted in medium as described above using a 1% methanol vehicle for all concentrations tested. Androgen assays used a dose response of testosterone as a positive control (EC_{50} f f i | 40 nM) and flutamide as a negative control (10^{-1} M; IC_{50} f f i | 200 nM, concentration required to suppress half the positive control activity), whereas estrogen assays used a dose response of 17ffl-estradiol as a positive control (EC_{50} f f i | 5 pM) and ICI 182,780 as a negative control (100 nM; IC_{50} f f i | 250 pM) (Supplemental Figure 1). The estrogen and androgen reporter gene assays have sensitivities within the ranges of those in other published studies, as reviewed previously (44). After induction for 18 to 24 hours, cells were incubated in a cell lysis solution for 20 minutes at 37°C before lysate was used for a luciferase reporter gene assay and ffi-Gal assay.

Hormonal activity was measured using a firefly luciferase reporter gene assay, as described previously (45). CMV-ffi-Gal activity was measured using a chlorophenol red-ffi-d-galactopyranoside substrate diluted to a concentration of 500 μ g/mL in a buffer consisting of 60 mmol/L sodium phosphate dibasic, 40 mmol/L sodium phosphate monobasic, 10 mmol/L potassium chloride, 1 mmol/L magnesium sulfate, and 50 mmol/L ffi-mercaptoethanol. The above mixture (200 μ L) was added to 20 μ L of cell lysate in a 96-well microtiter plate. Color was allowed to develop before the absorbance was read on a plate reader at a 570-nm wavelength.

CMV-ffi-Gal activity was used to normalize estrogen receptor assays but not used for androgen receptor assays. We found androgens to regulate CMV-ffi-Gal expression so did not use this to normalize the androgenic luciferase data. However, transfections were performed in flasks and then seeded into tissue culture plates, controlling for changes in transfection efficiency between wells. Thus, comparing the coefficients of variation (SD/mean) of normalized samples with those of un-normalized samples resulted in minimal change.

Sample toxicity

In MCF-7 cells, we used CMV-ffi-Gal activity as a marker of cell number. A serial 10-fold dilution of transfected cells was

used to assess the reliability of using CMV-ffi-Gal activity as a marker of cell number (r^2 f f i 0.996). As a result, we used this as a surrogate marker for sample toxicity, because estrogens were not found to regulate CMV-ffi-Gal expression. Thus, any sample found to have deviated significantly from the activity of the vehicle was deemed toxic and excluded from analysis. The following samples were excluded from analysis at the 40ffl concentration only: 1E, 3D, 5B, 5C, and 5E, whereas sample 3B was excluded at both the 4ffl and 40ffl concentrations for all assays. All samples were excluded from analysis at the 40ffl concentration within the androgenic assays due to observed cell-specific toxicity in the HepG-2 cell line. No evidence of toxicity was observed at the 4ffl concentration.

Calculation of estrogen/androgen receptor activities

Agonist activities were calculated as percent activity relative to the maximal positive control response of 100 pM 17ffl-estradiol and 10^{-1} M testosterone for estrogen and androgen receptor assays, respectively. Antagonist activities were calculated as percent suppression or enhancement of 10 pM estradiol or 100 nM testosterone, based on the EC_{50} values of the positive controls. Positive values denote additive agonist activities and negative values denote antagonist activities.

Statistical analysis

Linear mixed models (hierarchical linear models) were used to analyze the results from all three assays (estrogenic, antiestrogenic, and antiandrogenic), and incorporated random effects to account for dependence among measurements arising from the same sampling source within a site (Supplemental Figures 3–5). Fixed effects considered included site (Sites 1–5, Colorado River, Colorado reference, and Missouri reference), water type (ground/surface), concentration (40ffl/4ffl) and a covariate for the negative control of the assay plate, which was conceived as a baseline response for the assay. The Kenward-Roger method was used for estimating the degrees of freedom. Least-squares means, based on the final models, were used for planned contrasts and to compute 95% confidence intervals for differences of interest. A model selection criterion, the corrected Akaike information criterion, was used to evaluate relative goodness of fit of the models and therefore helped determine the final form of the model for the estrogenic assay. For ease of comparison and to avoid averaging over effects that may interact based on statistical results from the estrogenic assay, the same model form was used for the other assays when possible. Diagnostic plots were used to assess model fit and check distributional assumptions. PROC GLIMMIX in SAS 9.3 (SAS Inc.) was used for the data analysis.

Results

Estrogen and androgen receptor activities of chemicals used in natural gas operations

Antiestrogenic, antiandrogenic, and limited estrogenic activities were observed in the 12 natural gas drilling chemicals tested (Figure 2 and Supplemental Table 1), whereas no androgenic activity was observed. At 10^{-1} M,

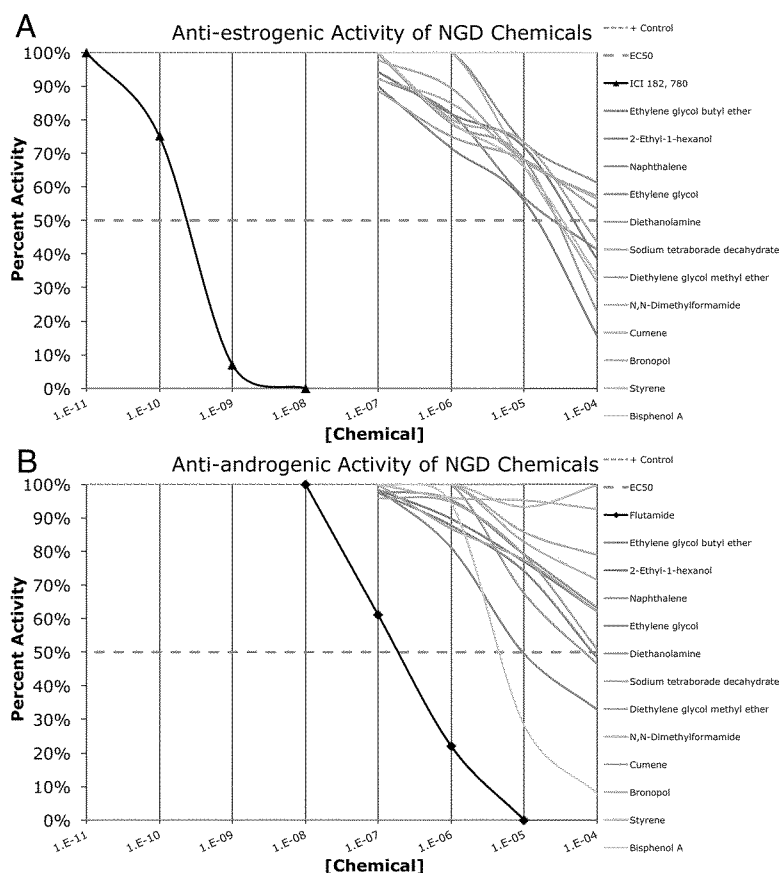


Figure 2. Estrogen and androgen receptor activities of selected chemicals used in natural gas operations. Representative dose responses of selected hydraulic fracturing chemicals tested for antiestrogenic (A) and antiandrogenic (B) activities. Antiestrogenic activity is presented as the percent suppression of 10 pM 17 β -estradiol (set to 100%) for each chemical from 0.1 to 100 μ M. Antiandrogenic activity is presented as the percent suppression of 100 nM testosterone (set to 100%) for each chemical from 0.1 to 100 μ M. NGD, natural gas drilling.

antiestrogenic activities ranged from 24% to 65% suppression of 10 pM 17 β -estradiol and antiandrogenic activities ranged from 0% to 63% suppression of 100 nM testosterone. The chemicals exhibited IC_{10} values (concentrations required to suppress 10% of the maximal activity of the positive control) ranging from 0.15 to 6.33 μ M (Figure 2). Of note, 2-ethyl-1-hexanol (IC_{10} ffi 0.60 μ M) and ethylene glycol (IC_{10} ffi 0.15 μ M) exhibited the greatest potencies for antiestrogenic activities and ethylene glycol (IC_{10} ffi 0.50 μ M), *N,N*-dimethylformamide (IC_{10} ffi 0.50 μ M), and cumene (IC_{10} ffi 0.62 μ M) exhibited the greatest potencies for antiandrogenic activities. Estrogenic activity was observed for bisphenol A, which exhibited supra-agonistic activity and an EC_{50} of 2.00 μ M (concentration required to exhibit half of its maximal activity). To our knowledge, this is the first report of the antiestrogenic activity of ethylene glycol monobutyl ether, 2-ethylhexanol, ethylene glycol, diethanolamine, diethylene glycol methyl ether, sodium tetraborate decahydrate, 1,2-bromo-2-nitropropane-1,3-diol, *N,N*-dimethylformamide, cumene, and styrene and the novel antiandrogenic activity of 2-ethylhexanol, naphthalene, dieth-

anolamine, sodium tetraborate decahydrate, 1,2-bromo-2-nitropropane-1,3-diol, and cumene.

Overall estrogen and androgen receptor activities of water samples

Surface and ground water samples were collected from sites 1 to 5 (sites in Garfield County with known natural gas drilling spills in a high-density natural gas drilling region), several locations along the Colorado River (the drainage basin for the entire drilling region), local reference sites in Garfield County with limited drilling activities nearby, and reference sites in Boone County, Missouri, an area devoid of natural gas drilling (Figure 1, Table 1, and Supplemental Table 2). Estrogenic, antiestrogenic, androgenic, and antiandrogenic activities were observed in 89%, 41%, 12%, and 46% of all water samples, respectively (Supplemental Figures 2 and 3). The types of activities observed differed widely among sites (Figure 3 and Supplemental Figures 2 and 3). Ground water at sites 1, 2, and 3 exhibited near-maximal estrogenic activities and low to moderate antiandrogenic activities, whereas both Garfield County and Missouri reference sites exhibited low levels of estrogenic activities only (Figure 3A). Surface water at sites 1 to 5 varied greatly; sites 1 and 4 exhibited low estrogenic, high antiestrogenic, and low to moderate antiandrogenic activities, sites 3 and 5 exhibited higher estrogenic and lower antiestrogenic activities, and site 2 exhibited only estrogenic activities (Figure 3B). The Colorado River samples exhibited activities at moderate levels, whereas the Missouri reference sites exhibited low estrogenic, very low antiestrogenic, and no antiandrogenic activities.

The results from all three assays were modeled using a mixed-model framework (Supplemental Figures 4, 5, and 6), with final model forms for the estrogenic and antiestrogenic assays consisting of a 3-way interaction (and all lower order terms) among the fixed effects (site, water type, and concentration), along with the baseline covariate (vehicle control). For the antiandrogenic assay, there was only one level of concentration used (4ffl), so 3-way interactions were not applicable. The antiandrogenic

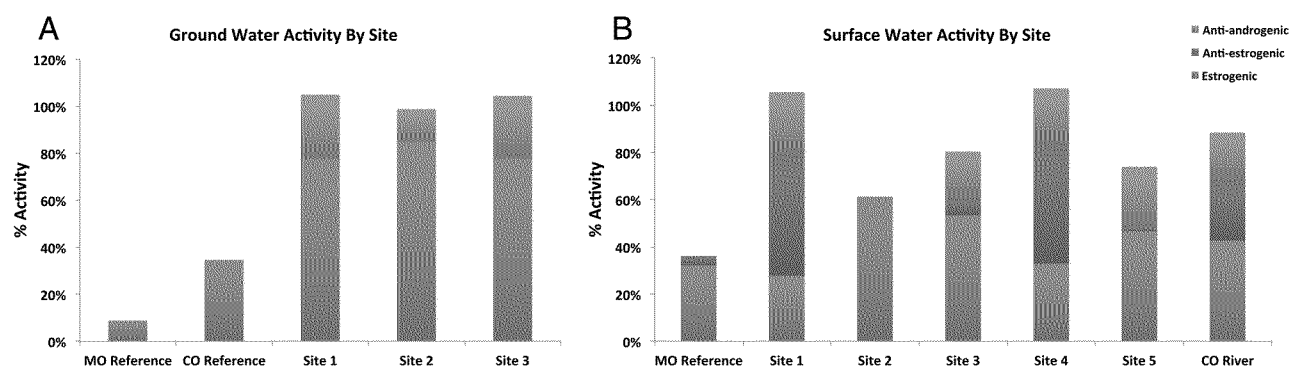


Figure 3. Combined estrogen and androgen receptor activities of ground and surface water by site. Combined estimated marginal means of estrogenic (blue), antiestrogenic (red), and antiandrogenic activities (green) at each sample collection site for ground water (A) and surface water (B). Estrogenic activities are expressed as a percentage of the activity of 100 pM 17 β -estradiol at 40ffl concentration, antiestrogenic activities are expressed as percent suppression of 10 pM 17 β -estradiol at 40ffl concentration, and antiandrogenic activities are expressed as percent suppression of 100 nM testosterone at 4ffl concentration. Antagonist activities are expressed as positive values; additive agonist activities are not expressed on this figure. The absence of a sample group for a particular figure panel is due to no samples present at that site for that particular water type. See Supplemental Table 2 for more details on each group.

model consisted of a site \times fl water type interaction term, main effect terms for site and water, and the baseline covariate (vehicle control).

Estrogenic activities of water samples from natural gas drilling—dense vs —sparse sites

Estrogenic activities were observed in both ground and surface water at sites 1 to 5 and in Colorado River samples. Low estrogenic activities were also observed in Garfield County and Missouri reference sites. Ground water samples collected from sites 1 to 3 exhibited higher estrogenic activities than both Garfield County and Missouri reference samples ($P = .0001$) (Figure 4A and Supplemental Tables 3 and 4). Interestingly, ground water samples collected from Garfield County reference sites exhibited higher estrogenic activities than Missouri reference sites ($P = .05$). Estrogenic activities tended to be higher in ground water samples than in surface water samples, with sites 1 to 5 exhibiting a minimum of 75% of maximal activity compared with a maximum of 60% in surface water samples. Surface water samples at sites 2, 3, and 5 exhibited greater estrogenic activities than the Missouri reference sites ($P = .05$) (Figure 4B and Supplemental Tables 3 and 4).

Antiestrogenic activities of water samples from natural gas drilling—dense vs —sparse sites

Antiestrogenic activity was observed in surface water at sites 1, 3, 4, and 5 and in Colorado River samples. Little to no antiestrogenic activity was observed in Garfield County or Missouri reference sites. Ground water samples exhibited little to no antiestrogenic activity, with sites 1 to 3 tending to exhibit greater additive agonist activities than reference sites (Figure 4C and Supplemental Tables 3 and 4), probably due to the high levels of estrogenic activities

exhibited by these samples (Figure 4A). Antiestrogenic activity was almost exclusively exhibited by surface water samples, where more apparent differences were observed between sites 1 to 5. Notably, sites 1 and 4 exhibited greater antiestrogenic activity than Missouri reference sites ($P = .05$) (Figure 4D and Supplemental Tables 3 and 4). The surface water samples collected from the Colorado River exhibited moderate activity, having less than site 4, which exhibited the highest antiestrogenic activity ($P = .05$) but not different from that for sites 1, 3, or 5. Site 2 displayed a clear absence of antiestrogenic activity.

Antiandrogenic activity of water samples from natural gas drilling—dense vs —sparse sites

Antiandrogenic activity was observed in ground and surface water at sites 1, 3, 4, and 5 and in Colorado River samples. No antiandrogenic activity was observed in Garfield County or Missouri reference sites. Water samples collected from sites 1 to 3 exhibited higher antiandrogenic activity than the Garfield County reference samples that exhibited additive agonist activity ($P = .01$) but did not differ from the Missouri reference sites that displayed no androgen receptor activity (Figure 4E and Supplemental Tables 3 and 4). Surface water samples collected from sites 1, 4, and 5 displayed greater antiandrogenic activity than the Missouri reference sites ($P = .05$) (Figure 4F and Supplemental Tables 3 and 4). Surface water samples collected from the Colorado River again displayed intermediate antiandrogenic activity that did not differ from antiandrogenic activity at sites 1 to 5 but that were significantly greater than the activity exhibited at the Missouri reference sites ($P = .05$). Site 2 displayed a clear absence of antiandrogenic activity.

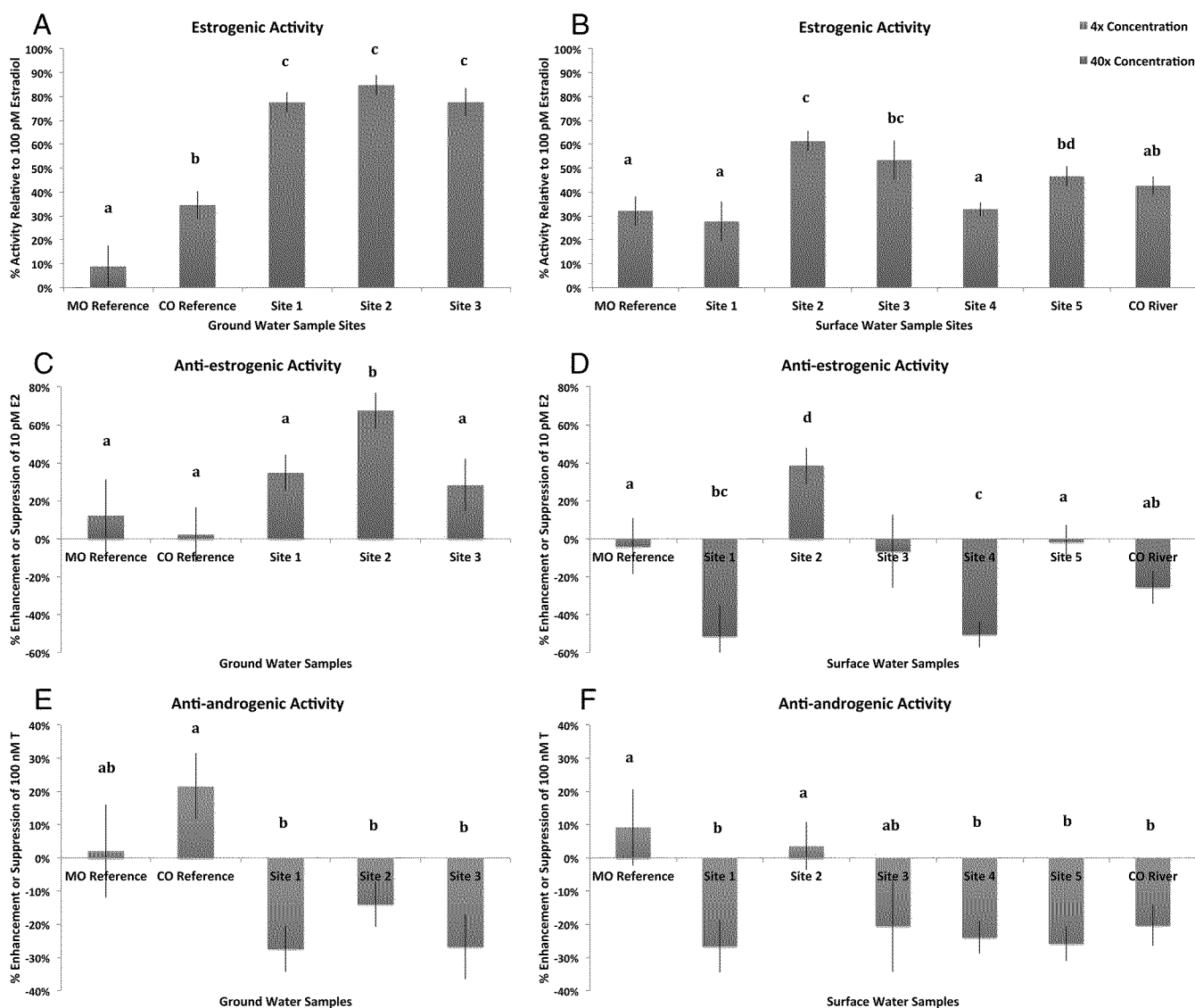


Figure 4. Average estrogen and androgen receptor activities of ground and surface water samples by site. Estimated marginal means \pm SEM of estrogenic activities of each ground water (A) and surface water site (B) relative to 100 pM 17 β -estradiol at 40ffl sample concentration. The estimated marginal means of antiestrogenic activities of each ground water (C) and surface water site (D) are expressed as percent suppression or enhancement of 10 pM 17 β -estradiol (set to 0) at 40ffl concentration. Negative values denote suppression of agonist activities and thus antagonist activities. Estimated marginal means of antiandrogenic activities of each ground water (E) and surface water site (F) as percent suppression or enhancement of 100 nM testosterone (set to 0) at 4ffl concentration. Negative values denote suppression of agonist activities and thus antagonist activities. Superscript letters denote statistical similarities and differences between sample groups within each pane. Groups containing the same letter were found to be the same, and groups with different letters were found to be significantly different. The absence of a sample group for a particular figure panel is due to no samples being present at that site for that particular water type. See Supplemental Table 2 for more details on each sample group.

Discussion

We report for the first time estrogenic, antiestrogenic, and antiandrogenic activity in a selected subset of chemicals used in natural gas operations and the presence of these activities in ground and surface water from a natural gas drilling-dense area in Garfield County, Colorado. One of 12 chemicals tested exhibited estrogenic activity, 11 had antiestrogenic activity, and 10 had antiandrogenic activity. Although these chemicals were selected because of their suspected or known EDC activity (19, 20), very few

had been shown to have direct receptor activity (44, 46–50). Thus, this is the first demonstration of antiestrogenic or antiandrogenic activity for most these chemicals.

Importantly, we found that water samples from sites with known natural gas drilling incidents had greater estrogen and androgen receptor activities than drilling-sparse or -absent reference sites. Very little estrogen or androgen receptor activity was measured in drilling-sparse reference water samples, moderate levels were measured in samples collected from the Colorado River (the

drainage basin for all Colorado collection sites), and moderate to high activities were measured in water samples from Garfield County spill sites. The Garfield County spill sites were known to have various types of contamination including produced water (wastewater and chemical mixture recovered after hydraulic fracturing), pipe leaks, a produced water tank spill, the improper disposal of produced water into surface water, and a natural gas upwelling (Table 1), which may have resulted in the distinct site-specific patterns of activities observed. At site 1, several ground water samples exhibited antiestrogenic activities despite the absence of antiestrogenic activities across all other ground water samples (Supplemental Figures 2 and 3). However, water quality testing performed at this site in September 2010 revealed high levels of mixing between surface and ground water, possibly explaining the notable differences observed (51). Site 2 exhibited an absence of antiestrogenic and antiandrogenic activities in contrast to those for the other spill sites. As described in Table 1, the spill at this site occurred into a creek and thus probably traveled away from the spill site more readily than at other sites, suggesting a basis for the different pattern of hormonal activities.

In the present study, we identified EDC activity of several individual chemical components used in natural gas operations that may contribute to the activity that we measured in water. Independent analyses identified these or similar chemicals at several of the sites we collected water from, despite the fact that our study did not pursue analytical identification of chemicals present in our water samples. At site 1, researchers at the University of Colorado collected water samples in September 2010 and performed analytical identification of chemicals present. Their testing revealed 5 polyethylene glycols used in natural gas drilling operations to be present in ground water from a monitoring well at this site (51). Our analysis of 3 ethylene glycols revealed antiestrogenic and antiandrogenic activities for ethylene glycol, ethylene glycol butyl ether, and diethylene glycol methyl ether. At site 5, an analytical laboratory found that water samples contained elevated levels of several BTEX (benzene, toluene, ethylbenzene, and xylenes) chemicals, which are reported to be associated with fracturing fluids (19–21). Naphthalene, which exhibited both antiestrogenic and antiandrogenic activity in the current study, was detected in soil samples collected from site 5 (52). Further, it was only detected at the site of the spill and not in the surrounding area, strongly suggesting that the source was the produced water leak.

Both naturally occurring chemicals and synthetic chemicals from other sources could contribute to the activity observed in the water samples collected in this study

(53–56). Although agricultural and animal care operations could potentially contribute to the measured activity in Garfield County, all sample sites were on land devoid of any recent animal care or agricultural use so these sources are likely to have minimal contributions. Wastewater contamination is another potential source of EDCs, and we acknowledge that Missouri reference samples were collected in an area that was more urban than the area for the Colorado samples (the Boone County population is approximately 3 times greater than the Garfield County population). However, because the Garfield County samples were all collected in more rural areas, we expect that any potential contribution through wastewater contamination would be lower in these samples. Further, the more urban samples were found to exhibit the lowest levels of hormonal activity in the current study. Taken together with results for independent analytical identification of drilling-related chemicals at sites we sampled from, this result provides further support for a link to the source of the activity observed.

Exposure to EDCs has been linked to a number of negative health outcomes in laboratory animals, wildlife, and humans (2, 12–17). Despite an understanding of adverse health outcomes associated with exposure to EDCs, research on the potential health implications of exposure to chemicals used in hydraulic fracturing is lacking. Bamberger and Oswald (26) analyzed the health consequences associated with exposure to chemicals used in natural gas operations and found respiratory, gastrointestinal, dermatologic, neurologic, immunologic, endocrine, reproductive, and other negative health outcomes in humans, pets, livestock, and wildlife species. Of note, site 4 in the current study was used as a small-scale ranch before the produced water spill in 2004. This use had to be discontinued because the animals no longer produced live offspring, perhaps because of the high antiestrogenic activity observed at this site. There is evidence that hydraulic fracturing fluids are associated with negative health outcomes, and there is a critical need to quickly and thoroughly evaluate the overall human and environmental health impact of this process. It should be noted that although this study focused on only estrogen and androgen receptors, there is a need for evaluation of other hormone receptor activities to provide a more complete endocrine-disrupting profile associated with natural gas drilling.

In conclusion, most water samples from sites with known drilling-related incidents in a drilling-dense region of Colorado exhibited more estrogenic, antiestrogenic, and/or antiandrogenic activities than the water samples collected from reference sites and 12 chemicals used in drilling operations exhibited similar activities. Taken together, the following support an association between nat-

ural gas drilling operations and EDC activity in surface and ground water: hormonal activities in Garfield County spill sites and the Colorado River are higher than those in reference sites in Garfield County and in Missouri, selected drilling chemicals displayed activities similar to those measured in water samples collected from a drilling-dense region, several of these chemicals and similar compounds were detected by other researchers at our sample collection sites, and known spills of natural gas fluids occurred at these spill sites. Taken together, this suggests that natural gas drilling operations may result in elevated EDC activity in ground and surface water.

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References

1. Yang M, Park MS, Lee HS. Endocrine disrupting chemicals: human exposure and health risks. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2006;24:183–224.
2. Tyler CR, Jobling S, Sumpter JP. Endocrine disruption in wildlife: a critical review of the evidence. *Crit Rev Toxicol*. 1998;28:319–361.
3. Jansen MS, Nagel SC, Miranda PJ, Lobenhof EK, Afshari CA, McDonnell DP. Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. *Proc Natl Acad Sci USA*. 2004;101:7199–7204.
4. Chen J, Ahn KC, Gee NA, et al. Triclocarban enhances testosterone action: a new type of endocrine disruptor? *Endocrinology*. 2008;149:1173–1179.
5. Hayes TB. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proc Natl Acad Sci USA*. 2002;99:5476–5480.
6. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ Health Perspect*. 2003;111:994–1006.
7. Myers JP, Zoeller RT, vom Saal FS. A clash of old and new scientific concepts in toxicity, with important implications for public health. *Environ Health Perspect*. 2009;117:1652–1655.
8. vom Saal FS, Akingbemi BT, Belcher SM, et al. Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. *Reprod Toxicol*. 2007;24:131–138.
9. Vandenberg LN, Colborn T, Hayes TB, et al. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev*. 2012;33:378–455.
10. Guillette LJ Jr, Gross TS, Masson GR, Matter JM, Percival HF, Woodward AR. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ Health Perspect*. 1994;102:680–688.
11. Ottinger MA, Lavoie E, Thompson N, et al. Neuroendocrine and behavioral effects of embryonic exposure to endocrine disrupting chemicals in birds. *Brain Res Rev*. 2008;57:376–385.
12. Sumpter JP, Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ Health Perspect*. 1995;103(suppl 7):173–178.
13. Kidd KA, Blanchfield PJ, Mills KH, et al. Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci USA*. 2007;104:8897–8901.
14. Kelce WR, Wilson EM. Environmental antiandrogens: developmental effects, molecular mechanisms, and clinical implications. *J Mol Med (Berl)*. 1997;75:198–207.
15. Akingbemi BT, Hardy MP. Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. *Ann Med*. 2001;33:391–403.
16. Mendiola J, Stahlhut RW, Jørgensen N, Liu F, Swan SH. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. *Environ Health Perspect*. 2011;119:958–963.
17. Christiansen S, Scholze M, Axelstad M, Boberg J, Kortenkamp A, Hass U. Combined exposure to anti-androgens causes markedly increased frequencies of hypospadias in the rat. *Int J Androl*. 2008;31:241–248.
18. Lind PM, Oberg D, Larsson S, Kyle CE, Orberg J, Rhind SM. Pregnant ewes exposed to multiple endocrine disrupting pollutants through sewage sludge-fertilized pasture show an anti-estrogenic effect in their trabecular bone. *Sci Total Environ*. 2010;408:2340–2346.
19. Waxman HA, Markey EJ, DeGette D. Chemicals used in hydraulic fracturing. US House of Representatives Committee on Energy and Commerce Minority Staff. <http://democrats.energycommerce.house.gov/sites/default/files/documents/Hydraulic-Fracturing-Chemicals-2011-4-18.pdf>. Accessed September 3, 2013.
20. Colborn T, Kwiatkowski C, Schultz K, Bachran M. Natural gas operations from a public health perspective. *Hum Ecol Risk Assess*. 2010;17:1039–1056.
21. Wiseman HJ. Untested waters: the rise of hydraulic fracturing in oil and gas production and the need to revisit regulation. *Fordham Environ Law Rev*. 2009;20:115–171.
22. Deutch J, Holditch S, Krupp F, et al. The SEAB shale gas production sub-

- committee ninety-day report—August 11, 2011. http://www.shalegas.energy.gov/resources/081111_90_day_report.pdf. Accessed September 3, 2013.
23. Riedl J, Rotter S, Faetsch S, Schmitt-Jansen M, Altenburger R. Proposal for applying a component-based mixture approach for ecotoxicological assessment of fracturing fluids. *Environ Earth Sci*. 2013;70:3907–3920.
 24. Lee DS, Herman JD, Elsworth D, Kim HT, Lee HS. A critical evaluation of unconventional gas recovery from the Marcellus shale, northeastern United States. *KSCE J Civil Eng*. 2011;15:679–687.
 25. DiGiulio DC, Wilkin RT, Miller C, Oberley G. 2011 *Investigation of Ground Water Contamination Near Pavillion, WY*. Ada, OK: US Environmental Protection Agency Office of Research and Development; 2011. Report no. EPA 600/R-00/000.
 26. Bamberger M, Oswald RE. Impacts of gas drilling on human and animal health. *New Solutions*. 2012;22:51–77.
 27. Kels S. State oil and gas agency groundwater investigations and their role in advancing regulatory reforms—a two-state review: Ohio and Texas. Ground Water Protection Council. <http://www.gwpc.org/sites/default/files/State%20Oil%20%26%20Gas%20Agency%20Groundwater%20Investigations.pdf>. Accessed September 3, 2013.
 28. Rozell DJ, Reaven SJ. 2011 Water pollution risk associated with natural gas extraction from the Marcellus Shale. *Risk Anal*. 2012;32:1382–1393.
 29. Osborn SG, Vengosh A, Warner NR, Jackson RB. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. *Proc Natl Acad Sci USA*. 2011;108:8172–8176.
 30. Jackson RB, Vengosh A, Darrah TH, et al. Increased stray gas abundance in a subset of drinking water wells near Marcellus shale gas extraction. *Proc Natl Acad Sci USA*. 2013;110:11250–11255.
 31. Fontenot BE, Hunt LR, Hildenbrand ZL, et al. An evaluation of water quality in private drinking water wells near natural gas extraction sites in the Barnett shale formation. *Environ Sci Technol*. 2013;47:10032–10040.
 32. Warner NR, Jackson RB, Darrah T, et al. Geochemical evidence for possible natural migration of Marcellus Formation brine to shallow aquifers in Pennsylvania. *Proc Natl Acad Sci USA*. 2012;109:11961–11966.
 33. Conservation Commission. Colorado weekly and monthly oil and gas statistics. <http://cogcc.state.co.us/Library/Statistics/CoWklyMnthlyOGStats.pdf>. Accessed September 5, 2013.
 34. Silva E, Rajapakse N, Kortenkamp A. Something from “nothing”—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ Sci Technol*. 2002;36:1751–1756.
 35. Christiansen S, Scholze M, Dalgaard M, et al. Synergistic disruption of external male sex organ development by a mixture of four anti-androgens. *Environ Health Perspect*. 2009;117:1839–1846.
 36. Soto AM, Calabro JM, Prechtl NV, et al. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in Eastern Nebraska, USA. *Environ Health Perspect*. 2004;112:346–352.
 37. Zhao JL, Ying GG, Yang B, et al. Screening of multiple hormonal activities in surface water and sediment from the Pearl River system, South China, using effect-directed in vitro bioassays. *Environ Toxicol Chem*. 2011;30:2208–2215.
 38. Waters Corporation. Endocrine disruptors—general SPE guidelines. <http://www.waters.com/webassets/cms/library/docs/oasis73.pdf>. Accessed September 3, 2013.
 39. Litvinov IV, Chang C, Isaacs JT. Molecular characterization of the commonly used human androgen receptor expression vector, pSG5-AR. *Prostate*. 2004;58:319–324.
 40. MacGregor GR, Caskey CT. Construction of plasmids that express *E. coli* β -galactosidase in mammalian cells. *Nucleic Acids Res*. 1989;17:2365.
 41. Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science*. 1988;240:327–330.
 42. Huang W, Shostak Y, Tarr P, Sawyers C, Carey M. Cooperative assembly of androgen receptor into a nucleoprotein complex that regulates the prostate-specific antigen enhancer. *J Biol Chem*. 1999;274:25756–25768.
 43. Nagel SC, Hagelbarger JL, McDonnell DP. Development of an ER action indicator mouse for the study of estrogens, selective ER modulators (SERMs), and Xenobiotics. *Endocrinology*. 2001;142:4721–4728.
 44. Thayer KA, Belcher SM. Mechanisms of action of bisphenol a and other biochemical/molecular interactions. World Health Organization and Food and Agriculture Organization of the United Nations. http://www.who.int/foodsafety/chem/chemicals/5_biological_activities_of_bpa.pdf. Accessed September 3, 2013.
 45. Brasier AR, Tate JE, Habener JF. Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. *Biotechniques*. 1989;7:1116–1122.
 46. Thomas P, Budiantara L. Reproductive life history stages sensitive to oil and naphthalene in Atlantic croaker. *Marine Environ Res*. 1995;39:147–150.
 47. Chang HY, Shih TS, Guo YL, Tsai CY, Hsu PC. Sperm function in workers exposed to *N,N*-dimethylformamide in the synthetic leather industry. *Fertil Steril*. 2004;81:1589–1594.
 48. Creasy D. A quantitative study of stage-specific spermatocyte damage following administration of ethylene glycol monomethyl ether in the rat. *Exp Mol Pathol*. 1985;43:321–336.
 49. Devillers J, Chezeau A, Thybaud E, et al. Ecotoxicity of ethylene glycol monobutyl ether and its acetate. *Toxicol Mech Methods*. 2002;12:255–263.
 50. Sohoni P, Sumpter JP. Several environmental oestrogens are also anti-androgens. *J Endocrinol*. 1998;158:327–339.
 51. Hill M. Effects of natural gas production on water quality in Garfield County, Western Colorado. *Honors J Univ Colorado-Boulder* 2011;1.
 52. Accutest Laboratories. (2010) Technical report for Accutest. Wheat Ridge, CO: Mountain States Accutest Laboratories; 2010.
 53. Kolpin DD, Furlong ET, Meyer MT, et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ Sci Technol*. 2002;36:1202–1211.
 54. Sychrová E, Štěpánková T, Nováková K, Bláha L, Giesy JP, Hilscherová K. Estrogenic activity in extracts and exudates of cyanobacteria and green algae. *Environ Int*. 2012;39:134–140.
 55. Murk AJ, Legler J, van Lipzig M, et al. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. *Environ Toxicol Chem*. 2002;21:16–23.
 56. Barnes KK, Kolpin DW, Furlong ET, Zaugg SD, Meyer MT, Barber LB. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States—I) Groundwater. *Sci Total Environ*. 2008;402:192–200.